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The Biosynthesis of Dicoumarol

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Micro-organisms have been isolated that can utilize *o*-coumaric acid as a sole carbon source with the subsequent production of 4-hydroxycoumarin and dicoumarol. One of these organisms, *Penicillium jenseni*, has been used to examine the biosynthesis of dicoumarol. Certain thermophilic fungi have also been found that can convert *o*-coumaric acid into dicoumarol.

Sweet clover (*Melilotus alba* or *Melilotus officinalis*) is used as a fodder crop in the form of hay or silage in many parts of the world. During storage the hay or silage may become 'spoiled', the term spoiled being used to denote the appearance of mould growth and the concurrent development of haemorrhagic properties. These haemorrhagic properties were shown by Stahmann, Huebner & Link (1941) to be due to the presence of dicoumarol (I). Similar spoilage has been shown to occur with hay from *Anthoxanthum odoratum* (Davies & Ashton, 1964).

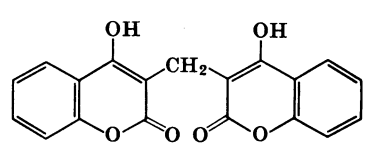
Neither *Melilotus alba* nor *Anthoxanthum odoratum* normally contains dicoumarol, and it has been postulated that the compound is formed by the micro-organisms present during spoilage. Smith & Brink (1938) suggested that coumarin (II) was the precursor of dicoumarol. It was further suggested (Stahmann *et al.* 1941) that 4-hydroxycoumarin (III) was a probable intermediate, since this compound is readily converted into dicoumarol in the presence of formaldehyde. These workers also explained on theoretical grounds that the relatively small amount of dicoumarol present compared with the large amount of coumarin was probably due to the 4-position of coumarin being the least likely site for direct hydroxylation. Gorz & Haskins (1964) have since shown that free coumarin is not present in large amounts in sweet clover. Mead, Smith & Williams (1958), reporting on the metabolism of coumarin and *o*-coumaric acid (IV) by rabbits, showed that 4-hydroxycoumarin was a metabolite of *o*-coumaric acid but not of coumarin. *o*-Coumaric acid is found in small amounts in sweet clover. In a preliminary report Bellis (1958) demonstrated that the moulds *Penicillium jenseni* and *Pen. nigricans* could form dicoumarol and 4-hydroxycoumarin from *o*-coumaric acid, but not from coumarin. This has been confirmed by Davies & Ashton (1964) using hay from oat grass (*Arrhenatherum elatius*) and cocksfoot (*Dactylis glomerata*) inoculated with *Pen. jenseni* and *o*-coumaric acid.

The microbial flora of sweet-clover hay, either before or after spoilage, has not been reported. The present work describes the isolation of soil micro-organisms that can produce dicoumarol. One of these organisms has been used to investigate further the biosynthetic pathway. Because of the high temperature, about 60°, developed in sweet-clover hay during spoilage (Linton, Goplen, Bell & Jaques, 1963), thermophilic organisms have also been utilized in biosynthetic studies.

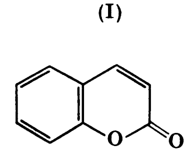
EXPERIMENTAL

Materials. Compounds used for microbiological work were of analytical grade except for coumarin and *o*-coumaric acid, which were of reagent grade recrystallized from ethanol. Water was glass-distilled and glassware was cleaned in chromic acid and rinsed with distilled water until free from acid.

The mineral-salts medium used had the following



(I)



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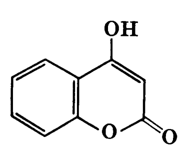

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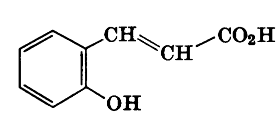

(IV)

composition: KCl (0·5g.), $NaNO_3$ (3·0g.), KH_2PO_4 (1·0g.), $MgSO_4,7H_2O$ (0·5g.) and water to 1000ml. Media were sterilized by heating at 116° for 30min. in an autoclave.

Pseudomonas fluorescens 5940 and *Ps. pyocyanea* 319, 1650 and 1999 were obtained from the National Collection of Type Cultures. *Humicolor stellata* 77024, *H. languinosa* 91791 and *Mucor pusillus* 96211 were obtained from the Commonwealth Mycological Institute.

Isolation of micro-organisms. Initially attempts were made to isolate organisms that could use coumarin as a sole carbon source. Although *o*-coumaric acid was later shown to be the precursor of dicoumarol it was found that several organisms that utilize coumarin can also metabolize *o*-coumaric acid.

Topsoil, from a number of widely differing localities, was scooped into sterilized screw-capped jars. About 10g. was removed from each jar with a sterilized spoon and transferred to 50ml. of nutrient broth (for bacteria) or sterile water (for moulds), shaken gently for 30min. and allowed to settle. Small volumes (0·25–1·0ml.) of the supernatant liquid were transferred to suitable media for incubation.

To isolate bacteria the supernatant was transferred to mineral-salts–agar plates with a narrow gutter containing 0·01M-coumarin in mineral salts–agar. After incubation at 24° for 8 days colonies growing on or near the gutter were removed and subcultured on nutrient agar.

To isolate moulds, potato–glucose–agar containing 0·1% of tannic acid to inhibit bacterial growth was used (Henderson & Farmer, 1955). The tannic acid allows the location of organisms with oxidizing properties, since these organisms produce a brown zone due to oxidation of the tannic acid. After subculturing to isolate individual organisms samples were transferred to mineral-salts medium containing 0·01% of coumarin.

Sweet-clover seed (*Melilotus alba*), obtained from Canada, was also examined for surface contaminants by first grinding the seeds and then incubating as before. In addition several typed organisms were examined for their ability to utilize coumarin as a sole carbon source. These included *Ps. pyocyanea* 319, 1650 and 1999, *Ps. fluorescens* 5940, *H. stellata* 77024, *H. languinosa* 91791 and *Mucor pusillus* 96211. Several pseudomonads were studied by Stanier (1948), who showed their ability to metabolize compounds such as benzoic acid as sole source of carbon.

General culture method. Mineral-salts medium (500ml.) containing the compound under investigation, with or without the addition of 2% of sucrose, was inoculated with the test organism. Most organisms grow only slowly with coumarin or *o*-coumaric acid as the sole carbon source. Consequently in certain experiments with moulds where larger amounts of mycelium were required the spores were first transferred to mineral-salts medium containing 2% of sucrose. After incubation for 7 days the dense mycelial mat was aseptically washed and transferred to the appropriate culture medium.

Flasks were incubated at 24°, except for thermophilic fungi, with intermittent shaking. Samples of the media were taken at intervals and examined for metabolites. Culture media with coumarin as a sole carbon source contained 0·01% of coumarin, whereas *o*-coumaric acid was used at concentrations of either 0·03% or 0·005%.

The thermophilic fungi were grown in a mineral-salts medium containing 0·005% of *o*-coumaric acid plus 0·02% of sucrose. *H. stellata* required the addition of 0·04% of yeast extract (Oxoid). Incubation was at 40° with oxygen aeration at the rate of about 15ml./min.

Isolation of metabolites. Samples of the media were filtered free from mycelial debris and shaken mechanically for 20min. with an equal volume of chloroform. The chloroform layer was separated and evaporated to about 1ml.

Identification of metabolites. Compounds were identified by their chromatographic mobilities, colour reactions and u.v.-absorption spectra.

Paper chromatograms were run on Whatman 3MM paper, descending technique, with the following solvent systems: (1) ethylene glycol–chloroform + HCl atmosphere; (2) ethylene glycol–ethylene dichloride; (3) propylene glycol–di-*n*-butyl ether; (4) formamide–chloroform + HCl atmosphere; (5) chloroform–formic acid (19:1, v/v); (6) *n*-butanol–1·5N-ammonia (1:1, v/v; organic phase). For partition chromatography (solvents 1–4) the paper was first immersed in a 30% (v/v) solution of the stationary phase (ethylene glycol or formamide) in acetone. The paper was blotted between sheets of filter paper and the acetone removed by evaporation in an air draught. In solvents 1 and 4 the HCl atmosphere was provided by a beaker containing 10N-HCl in the bottom of the tank. Pure authentic compounds were used as markers in all chromatographic systems. The resulting separations were examined for fluorescence in u.v. light at 350 and 254mμ in the presence of ammonia vapour. The paper was then sprayed with either N-NaOH or 0·1% Brentamine Fast Blue B salt solution.

Spots corresponding in position to dicoumarol and 4-hydroxycoumarin were eluted from the paper with chloroform and ethanol respectively. The eluates were evaporated to dryness and the residues redissolved in 5ml. of ethanol (4-hydroxycoumarin) or 5ml. of 2·5N-NaOH (dicoumarol). The u.v.-absorption spectra of these solutions were examined and compared with those of authentic compounds with a Perkin–Elmer 137 spectrophotometer.

Quantitative conversion of o-*coumaric acid into 4-hydroxycoumarin and dicoumarol.* Samples of the media, which had been incubated for 14 days, were extracted with chloroform, then chromatographed and quantitatively analysed for dicoumarol by the method of Christensen (1964). The total 4-hydroxylated coumarins (4-hydroxycoumarin and dicoumarol) were determined by heating another sample under reflux for 10min. with formaldehyde, which converted 4-hydroxycoumarin quantitatively into dicoumarol.

RESULTS

The identities of the biosynthetically derived 4-hydroxycoumarin and dicoumarol were confirmed by comparison of the R_F values (Table 1), colour reactions on chromatograms (Table 2) and u.v.-absorption spectra (Figs. 1 and 2) with authentic samples of 4-hydroxycoumarin and dicoumarol.

Examinations of soil micro-organisms able to utilize coumarin as a sole carbon source yielded two bacteria and two moulds. The moulds have been identified as *Pen. jenseni* and *Pen. nigricans.* The ability of these two moulds and bacteria, and also the typed organisms, to grow on coumarin or *o*-coumaric acid media is reported in Table 3. This

Table 1. *Chromatographic mobilities of coumarin,* o-*coumaric acid, 4-hydroxycoumarin, 7-hydroxycoumarin and dicoumarol*

Details of solvents are given in the text. d, Diffuse; —, not determined. A, Authentic compounds; B, biosynthetic compound (*Pen. jenseni*).

	R_F values					
Compound Solvent ...	1	2	3	4	5	6
Coumarin	d	d	0·57	d	0·94	0·90
o-Coumaric acid	0·03	0·08	0·10	0·06	0·48	0·20
7-Hydroxycoumarin	0·25	—	0·12	0·34	—	—
4-Hydroxycoumarin (A)	0·31	0·16	0·10	0·39	0·70	0·40
4-Hydroxycoumarin (B)	0·30	0·12	0·10	0·37	0·68	0·44
Dicoumarol (A)	0·95	0·90	0·13	0·98	0·94	0·80
Dicoumarol (B)	0·94	0·87	0·14	0·95	0·95	0·82

Table 2. *Colour reactions and ultraviolet-absorption characteristics of coumarin,* o-*coumaric acid, 4-hydroxycoumarin, 7-hydroxycoumarin and dicoumarol*

—, No colour or fluorescence. A, Authentic compound; B, biosynthetic compound (*Pen. jenseni*).

	Ultraviolet light				Ultraviolet absorption in ethanol	
Compound	254 mμ + NH_3 vapour	350 mμ + NH_3 vapour	350 mμ + NaOH	Brentamine Fast Blue B salt	$\lambda_{max.}$	$\log\epsilon_{max.}$
Coumarin	—	—	Green	—	275	4·0
o-Coumaric acid	Green	Green	Green	Yellow	274	4·22
4-Hydroxycoumarin (A and B)	Quenches	Quenches	Quenches	Red	268	4·0
7-Hydroxycoumarin	Blue	Blue	Blue	Yellow	330	4·05
Dicoumarol (A and B)	—	—	—	Orange	317*	4·42*

* In 2·5N-NaOH.

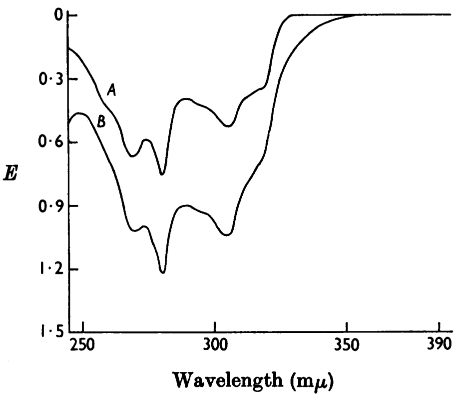

Fig. 1. Ultraviolet-absorption spectra of synthetic (*A*) and biosynthetic (*B*) 4-hydroxycoumarin (*A* contains 11·6 μg./ml.).

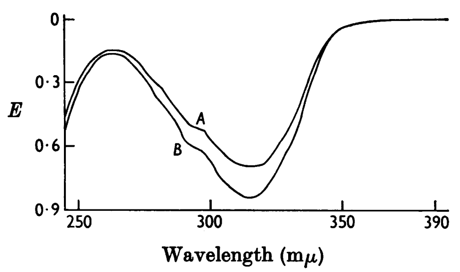

Fig. 2. Ultraviolet-absorption spectra of synthetic (*A*) and biosynthetic (*B*) dicoumarol (*A* contains 9·35 μg./ml.).

Table also records the formation of dicoumarol from these carbon sources.

The quantitative conversion of *o*-coumaric acid into 4-hydroxycoumarin and dicoumarol is recorded in Table 4. After 14 days about 60% of the added *o*-coumaric acid was converted into these two compounds.

DISCUSSION

The theoretical considerations of electron densities at the various carbon atoms in the coumarin

Table 3. *Ability of certain micro-organisms to utilize coumarin or* o-*coumaric acid as sole carbon sources with the concurrent production of dicoumarol*

Experimental details are given in the text.

Micro-organism	Coumarin media: Growth	Coumarin media: Dicoumarol production	*o*-Coumaric acid media: Growth	*o*-Coumaric acid media: Dicoumarol production
Ps. pyocyanea 319	−	−	−	−
Ps. pyocyanea 1999	−	−	−	−
Ps. pyocyanea 1650	−	−	−	−
Ps. fluorescens 5940	−	−	−	−
Soil bacterium (coccus)	+	−	+	−
Soil bacterium (rod)	+	−	+	−
Soil mould (*Pen. jenseni*)	+	−	+	+
Soil mould (*Pen. nigricans*)	+	−	+	+
H. stellata	+	−	+	+
H. lanuginosa	+	−	+	+
Mucor pusillus	+	−	+	+

Table 4. *Quantitative conversion of* o-*coumaric acid into 4-hydroxycoumarin and dicoumarol*

o-Coumaric acid at start (μg.)	Dicoumarol isolated (μg.)	4-Hydroxycoumarin and dicoumarol isolated (as dicoumarol) (μg.)	4-Hydroxycoumarin isolated (μg.) (calc.)
1000	334	630	296
1000	304	615	311

molecule indicated that electrophilic substitution of coumarin in the 4-position would be difficult (Samuel, 1955). Bellis (1960), using γ-irradiation, Fenton's reagent, alkaline peroxide and peroxidase under a wide variety of conditions, was unable to demonstrate any 4-hydroxylation of coumarin *in vitro*; the 7-hydroxy compound (umbelliferone) was produced in many experiments. This suggests that coumarin is an unlikely precursor of 4-hydroxycoumarin and dicoumarol. Mead *et al.* (1958) examined the metabolism of coumarin by rabbits and identified all possible hydroxycoumarins except 4-hydroxycoumarin.

Two of the moulds, identified as *Pen. jenseni* and *Pen. nigricans*, and two bacteria isolated from soil samples were able to utilize coumarin as a sole carbon source. The three thermophilic fungi were also able to metabolize coumarin. However, in none of these experiments was dicoumarol or 4-hydroxycoumarin identified in the culture media. When grown on *o*-coumaric acid as the sole carbon source all the above-mentioned organisms, except the two bacteria, produced dicoumarol and 4-hydroxycoumarin. The formation of dicoumarol by all three of the thermophilic fungi examined is significant, since spoiled hay may attain a temperature of 55–60°, which may be maintained for several days (Linton *et al.* 1963). Such high-temperature conditions would greatly stimulate the growth of thermophilic organisms. *H. stellata, H. lanuginosa* and *Mucor pusillus* are all present in hay (Cooney & Emerson, 1964).

During the metabolism of *o*-coumaric acid by *Pen. jenseni* 4-hydroxycoumarin can be identified in the culture medium long before the appearance of dicoumarol. 4-Hydroxycoumarin can readily be converted into dicoumarol by treatment with formaldehyde at room temperature (Anschütz, 1909). Air-pollution studies (Lawson, 1951) have shown formaldehyde to be present in the atmosphere. Formaldehyde has also been detected in dew (Ram & Dhor, 1933). Since the appearance of dicoumarol in culture media containing 4-hydroxycoumarin is slow it is possible that the methylene bridge of dicoumarol is introduced non-enzymically, the rate being limited by the availability of exogenous formaldehyde.

The results presented in this paper support the view that the biosynthesis of dicoumarol is by way of *o*-coumaric acid and 4-hydroxycoumarin.

We thank Dr David Park for the identification of *Pen. jenseni* and *Pen. nigricans*, and the Department of Bacteriology for the strains of *Ps. pyocyanea*. We are also grateful to Dr B. P. Goplen, who supplied the *Melilotus alba* seed.

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